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REVIEW

Immunological considerations in the development of *Pseudomonas aeruginosa* vaccines

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic human pathogen capable of causing a wide range of potentially life-threatening infections. With multidrug-resistant *P. aeruginosa* infections on the rise, the need for a rationally-designed vaccine against this pathogen is critical. A number of vaccine platforms have shown promising results in pre-clinical studies, but no vaccine has successfully advanced to licensure. Growing evidence suggests that an effective *P. aeruginosa* vaccine may require Th17-type $CD4^+$ T cells to prevent infection. In this review, we summarize recent pre-clinical studies of *P. aeruginosa* vaccines, specifically focusing on those that induce Th17-type cellular immunity. We also highlight the importance of adjuvant selection and immunization route in vaccine design in order to target vaccine-induced immunity to infected tissues. Advances in cellular immunology and adjuvant biology may ultimately influence better *P. aeruginosa* vaccine platforms that can protect targeted human populations.

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Introduction to *P. aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, motile, rod-shaped bacterium that is ubiquitous in the environment. *P. aeruginosa* is a quintessential opportunistic pathogen and the etiologic agent of several potentially life-threatening infections, including health-care-associated and ventilator-associated pneumonia, chronic pulmonary infection in cystic fibrosis (CF) patients, and burn and soft tissue infections. The increasing prevalence of drug-resistant *P. aeruginosa* infections has prompted the World Health Organization to list *P. aeruginosa* as one of the top priorities for the development of new medical countermeasures[1].

The genome of *P. aeruginosa* contains a highly conserved core genome and a highly variable accessory genome, which encodes for a broad range of transporters, transcriptional regulators, and two-component regulatory systems.[2] The genetic diversity of *P. aeruginosa* provides metabolic versatility for the organism, allowing it to survive in a multitude of environments, ranging from soil and water to biofilms formed within catheters or ventilator equipment. Importantly, this genetic pliability also contributes to *P. aeruginosa*'s multidrug resistance. Current estimates by the CDC indicate that approximately 6700 multidrug resistant *P. aeruginosa* infections occur in the United States annually.[3]

P. aeruginosa uses a multi-faceted approach to survive within a host, including cell surface molecules that aid in attachment to host cells and bacterial secretion systems that produce toxins and effector proteins to evade or modulate the host immune response (ie. type three secretion systems). Once *P. aeruginosa* enters an immune-compromised individual, the bacterium uses flagella for motility and numerous type IV pili to mediate attachment to cell surfaces. The surface components of *P. aeruginosa*, including lipopolysaccharide (LPS) and the exopolysaccharide alginate, mediate bacterial adherence to host cells and facilitate bacterial survival within the host. Alginate is believed to protect the bacterium in harsh environments and from oxidative stress and immunological attack, such as that encountered in the CF lung.[4] Alginate also contributes to biofilm formation and enables *P. aeruginosa* to persist in the lungs of individuals with CF, leading to chronic infection, enhanced morbidity, and worsening prognosis for these patients.[4] The outer membrane of *P. aeruginosa* also contains several proteins that function to stabilize and protect the bacterium, including those that control or facilitate molecular transport across the membrane barrier. These proteins are highly conserved across *P. aeruginosa* serogroups and remain phenotypically stable during biofilm formation.[5] Collectively, these surface and secreted components can modulate the host immune system, damage host tissues, and dictate bacterial virulence. For these reasons, many of these components are the natural target of adaptive immune responses and have been explored as vaccine candidates. For a more comprehensive review of *P. aeruginosa* virulence factors and survival strategies in the human host, the reader is referred to Moradali et al.[6]

P. aeruginosa infections

The multitude of virulence factors possessed by *P. aeruginosa* contributes to its versatility and the diverse manifestations of disease associated with *P. aeruginosa* infections. *P. aeruginosa* is a significant human pathogen, capable of establishing infections in the respiratory tract, urinary tract, skin and soft tissues, eyes, and ears. Infections occur primarily in patients with physical, phagocytic, or immunologic defects in host defense mechanisms. As a nosocomial pathogen, *P. aeruginosa* infections pose a strenuous burden on the health care system and are responsible for 17% of ventilator associated pneumonias,[7] 9% of other

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healthcare associated pneumonias,[8] 10% of catheter-associated urinary tract infections, 4% of central line-associated blood stream infections, and 6% of surgical site infections.[9] *P. aeruginosa* is also the predominant bacteria infecting the lungs of CF patients, causing severe morbidity and mortality in these individuals.[10]

Additionally, *P. aeruginosa* is becoming an increasingly common pathogen isolated from personnel returning from Iraq and Afghanistan with combat-related infections.[11,12] Off the battlefield, *P. aeruginosa* continues to pose a challenge in burn wound infections, with antibiotic resistance rapidly increasing in this patient population.[13–15] Cancer patients who suffer from chemotherapy-induced neutropenia are also a growing clinical group at high-risk for *P. aeruginosa* infections, including pneumonia and bacteremia.[16–18] The diversity of clinical infections and increasing drug-resistance highlight the immense need for a rationally-designed vaccine against *P. aeruginosa*. The target population for a *P. aeruginosa* vaccine is broad, encompassing the elderly, those with underlying chronic lung diseases, and military personnel.

P. aeruginosa vaccines

Despite the clear morbidity and mortality associated with *P. aeruginosa*, no vaccine has ever been licensed for the prevention of infection. This is not for lack of effort. In 1970, Alexander and Fisher published a letter citing that a *P. aeruginosa* LPS-based vaccine prevented mortality in burn patients.[19] Since that publication, numerous attempts have been made to develop and advance a *P. aeruginosa* vaccine towards licensure. Historically, vaccine development for *P. aeruginosa* has focused on identification of protective antigens and utilization of various vaccine platforms, including live-attenuated or whole-cell inactivated strains, subunit, conjugate, and DNA vaccines. Some of these candidate vaccines produced very promising results in animal models, primarily based on protective antibodies, and were advanced to clinical trials. For detailed reviews of *P. aeruginosa* vaccine antigens and previous clinical trials, the reader is referred to Priebe and Goldberg,[20] Sharma et al.,[21] Worgall et al.,[22] Grimwood et al.,[23] and Merakou et al.[24] Despite these attempts, no vaccine is currently on the market. The failure of previous *P. aeruginosa* vaccines is likely multifactorial, yet it warrants a re-evaluation of the criteria by which former candidates were appraised and advanced. In this review, we will examine candidate *P. aeruginosa* vaccines from recent years (Table 1) and highlight the emerging importance of specific cellular immune responses in vaccine-mediated protection. First, it is important to review the role of humoral (antibody) and cellular immune responses in the context of bona fide *P. aeruginosa* infection.

Table 1. Pre-clinical *P. aeruginosa* vaccine studies that examine Th17 cellular immunity.

Study	Antigen/ adjuvant	Route	Model	Cellular Immune Response
Priebe et al. 2008[25]	PA14ΔaroA	Intranasal	Murine model of intranasally induced acute pneumonia	Protection was dependent upon T cell-secreted IL-17
Wu et al. 2012[26]	PopB and PcrH/curdlan	Intranasal	Murine model of intranasally induced acute pneumonia	Protection was antibody independent and correlated with enhanced mucosal IL-17 and Th17 responses
Kamei et al. 2012[27]	PA01ΔaroA	Intranasal	Neutropenic murine model of intranasally induced acute pneumonia	Vaccine efficacy was $CD4^+$ T cell dependent and pulmonary GM-CSF was critical and associated with an increase in IL-17
Krause et al. 2013[28]	Adenoviral vector expressing OprF with RGD capsid modification	Intratracheal	Murine intratracheal challenge with *P. aeruginosa* encapsulated in agar beads	Vaccination-induced reduction in pulmonary bacterial load was associated with an increase in IL-17, IL-4, and IL-5 production by stimulated lung $CD4^+$ T cells
Banadkoki et al. 2016[29]	PilA/alum + naloxone	Subcutaneous	Murine model of intranasally induced acute pneumonia	Vaccine-induced protection was associated with an increase in IL-17, IFN-γ, and IL-4 production by stimulated splenocytes
Korpi et al. 2016[30]	PilA and Type B flagellin	Subcutaneous	Murine burn wound sepsis model	Vaccine-induced protection was associated with an increase in IL-17, IFN-γ, and IL-4 production by stimulated splenocytes
Behrouz et al. 2016[31]	Type B flagellin/alum	Subcutaneous	Murine burn wound sepsis model	Vaccine-induced protection was associated with an increase in IL-17, IFN-γ, and IL-4 production by stimulated splenocytes
Li et al. 2016[32]	X-ray irradiated *P. aeruginosa*	Intranasal	Murine model of intranasally induced acute pneumonia	Vaccine-induced protection was dependent on CD4 + T cells and IL-17 production
Gao et al. 2017[33]	Recombinant OprL/ curdlan	Intranasal	Murine intratracheal induction of acute pneumonia	Vaccine-induced protection was associated with an increase in $CD4^+$ $IL17^+$ T cells in the lungs of mice after *P. aeruginosa* infection
Behrouz et al. 2017[34]	Bilvalent flagellin	Intranasal	Murine model of intranasally induced acute pneumonia	Vaccine-induced protection was dependent on IL-17
Schaefers et al. 2018[35]	PopB and PcrH encapsulated into PLGA* nanoparticles	Intranasal	Murine model of intranasally induced acute pneumonia	Vaccine-induced protection was associated with an increase in $CD4^+$ $IL17^+$ T cells in the lungs and increased IL-17 production by stimulated splenocytes
Bakht Azad et al. 2018[36]	PilQ and Type B-flagellin/alum	Subcutaneous	Burned mouse model	Vaccine-induced protection was associated with an increase in IL-17 and IL-4 production by stimulated splenocytes
Meynet et al. 2018[37]	Killed But Metabolically Active (KBMA) *P. aeruginosa*	Subcutaneous	Murine model of intranasally induced acute pneumonia	Vaccine-induced protection was associated with a mixed Th1/Th17-type $CD4^+$ T cell response
Baker et al. 2019[38]	Outer membrane proteins/dmLT	Intradermal	Murine oropharyngeal aspiration leading to acute pneumonia	Vaccine-induced protection was associated with a mixed Th1/Th17-type $CD4^+$ T cell response and an increase in IFN-γ and IL-17 production in the lungs after *P. aeruginosa* infection

*PGLA: poly-lactic-co-glycolic acid.

Immune responses to *P. aeruginosa*

Immunity to *P. aeruginosa* has been most extensively studied in CF patients. Once colonized with *P. aeruginosa*, CF patients mount antibody responses to many *P. aeruginosa* antigens.[39] CF adults who were not chronically colonized with *P. aeruginosa* possessed antibodies to alginate that were shown to mediate opsonophagocytosis,[39] indicating some protective potential. However, in most cases, antibodies are unable to sufficiently curb the spread of infection, suggesting that infection-induced antibodies do not confer sufficient protection against future *P. aeruginosa* infections in these patients. Clinical studies comparing CF patients with and without chronic infection observed that patients with persistent *P. aeruginosa* lung infection had an immune response predominantly of the Th2 type, whereas patients with the highest production of IFN-γ, a Th1 cytokine, had the best lung function, indicating that Th1 T cells may be essential mediators of protection.[40] Another study assessing CF patients chronically infected with *P. aeruginosa* found significantly higher levels of pulmonary Th2 cells and the Th2 cytokines IL-4, IL-13, and thymus and activation-regulated chemokine (TARC, also known as CCL17) in bronchoalveolar lavage fluid and lower levels of IFN-γ compared with uninfected patients with CF and healthy controls.[41] Bronchoalveolar lavage fluid levels of these Th2 cytokines correlated inversely with pulmonary function.[41] In a prospective study of children with CF, TARC was significantly increased in patients who developed *P. aeruginosa* infection during the 2 years of study.[42] Assessment of cytokine expression in mucosal bronchial biopsies of CF patients found the highest expression of TGF-β and IFN-γ in CF patients with only mild disease and a history of infrequent exacerbations, as compared to those patients with frequent acute exacerbations and chronic infection.[43] Despite the cumulative findings described above, it is important to note that CF patients can display tremendous heterogeneity in their immune response to *P. aeruginosa*.[40,44] Moreover, inherent defects in anti-bacterial mechanisms may diminish the effectiveness of adaptive immunity in the CF lung.[25,45] Thus, it is important to examine protective immunity to *P. aeruginosa* in other biological systems and non-CF models as well.

Animal models of pulmonary infection also demonstrate a protective role for Th1 cells. Resistance to re-infection with *P. aeruginosa* in mice was associated with a Th1 response, demonstrated by a higher IFN-γ/IL-4 ratio.[45] In a vaccine study utilizing a live-attenuated *P. aeruginosa* strain, passive transfer of purified IgG failed to protect mice against heterologous strain challenge, whereas active immunization was protective.[20,25] Additionally, mice that have a Th1 bias are better protected compared to mice with a clear Th2 bias.[40] These results suggest that cellular immunity, and in particular Th1 T cell immunity, may play a key role in protection against *P. aeruginosa* infection.

Additionally, Th17 cells have sparked significant research since their discovery, particularly due to their role in the mucosal immune response against pulmonary pathogens.[46–48] The multiple downstream effects of IL-17 indicate that the Th17 response strikes a precarious balance between protecting the mucosal surfaces and facilitating destructive tissue inflammation.[44] IL-17 regulates granulopoesis by regulating production of G-CSF and also actively recruits neutrophils to sites of infection through the induction of CXC cytokines at sites of inflammation.[47,49–51] IL-17 is also induced in the lung in response to mucoid *P. aeruginosa* infection. Significantly higher levels of IL-17 are found in bronchial secretions of CF patients following acute pulmonary exacerbations[42] and IL-17 has been shown to be required for the control of chronic *P. aeruginosa* pulmonary disease in mouse models.[52] Importantly, it is known that the secretion of IL-17A by $CD4^+$ T cells is essential for the rapid recruitment of neutrophils to the lungs.[25,53] Neutrophils are essential for the efficient killing of *P. aeruginosa* during acute pulmonary infection, indicating that Th17 cells, like Th1 cells, may be important for the complete control of *P. aeruginosa*.[54]

An emerging role for vaccine-induced cellular immunity

Both arms of the immune system may work in concert to protect against *P. aeruginosa* infection as growing evidence suggests that a successful *P. aeruginosa* vaccine must elicit both opsonizing antibodies[55,56] and $CD4^+$ T cells to provide complete protection against infection.[25,27] Despite an emerging role for cellular immunity in the host immune response to *P. aeruginosa*, only a handful of pre-clinical vaccine studies have closely scrutinized T-helper subsets elicited by vaccination (Table 1).

Examination of live, attenuated vaccines demonstrated that vaccine-induced protection against *P. aeruginosa* intranasal infection in mice was dependent on Th17 cells, as antibody-mediated depletion of IL-17 before challenge or absence of the IL-17 receptor abrogated vaccine-induced protection against bacterial challenge.[25] Further examination revealed that protection against *P. aeruginosa* pulmonary infection in a neutropenic mouse was dependent on Th17 T cells and that pulmonary GM-CSF was critical and associated with production of IL-17.[27] In an immune-competent mouse model, the production of IL-17 by $CD4^+$ T cells after immunization with an X-ray irradiated vaccine was associated with rapid recruitment of neutrophils to the lungs, resulting in protection against *P. aeruginosa*.[32]

A Th17-based reverse vaccinology strategy using a library of 258 *P. aeruginosa* outer membrane and secreted proteins identified several proteins, including outer membrane protein L (OprL), PopB, PcrH, and PilQ that induced protective memory responses.[26] In particular, PopB and PcrH combined with the adjuvant curdlan, conferred IL-17-dependent and antibody-independent protection from *P. aeruginosa* in an acute pneumonia model.[26] Further investigations of PopB, PcrH, OprL, and PilQ in several different vaccine formulations also demonstrated that vaccine-induced protection was associated with pulmonary $CD4^+$ T cells[33,35] and an increase in IL-17.[36]

A multitude of other protein-based and multicomponent vaccines have been examined over the last decade. Pili,[29,30] flagellin,[29,30,34] outer membrane proteins[28,38] and some whole cell vaccines[37] all demonstrate an ability to induce both Th1

and Th17-type immune responses either systemically or within the lungs. As discussed below, the ability of these vaccines to promote cellular immunity, and specifically a Th-17-type immune response, may be less dependent on the antigen and influenced more by the inclusion of Th-17-promoting adjuvants and/or the route of vaccination.

Vaccine strategies to target cellular immunity

Adjuvant selection

Although antigen discovery and selection is of considerable importance for rational vaccine design, adjuvant selection and examination is also critical. Adjuvants can act as pathogen-associated molecular patterns (PAMPs) by triggering the innate immune response, inducing the activation and maturation of antigen presenting cells, and subsequently initiating downstream adaptive immune responses to the associated vaccine antigens.[57] In doing so, adjuvants can also improve vaccine efficacy in populations where responses to vaccines are typically reduced, such as those with underlying immunodeficiencies.[58] Perhaps not surprisingly, the majority of *P. aeruginosa* pre-clinical and clinical vaccine trials utilized aluminum salts (alum) as the adjuvant or did not utilize an adjuvant. Although the exact mechanisms of alum adjuvanticity are not well understood, alum acts primarily to increase antibody production. It is an excellent option for vaccines targeting pathogens targeted primarily by antibodies. As it appears that $CD4^+$ T cells play a considerable role in protection against *P. aeruginosa*, alum may not be the optimal adjuvant when designing vaccines for this pathogen.

Of importance, depending on their mechanism of action, adjuvants can skew the cellular immune response towards a Th1, Th2, or Th17 response or some combination thereof. Several adjuvants have demonstrated the ability to enhance Th17 responses during vaccination, including muramyl dipeptide,[59] the bacterial ADP-Ribosylating Enterotoxin Adjuvant (BARE) double mutant of *E. coli* heat-labile toxin (dmLT),[38,60] monophospholipid A (MPL),[61] and curdlan.[62] Indeed, addition of dmLT to an OMP-based *P. aeruginosa* vaccine significantly increased the production of IL-17 by $CD4^+$ T cells in the lungs of protected mice.[38] Similar results were achieved with the addition of curdlan adjuvant to a recombinant OprL-based vaccine.[33]

An additional advantage of promoting a Th17-based vaccine response is the potential that immunocompromised individuals who do not develop adequate antibody responses, including high risk populations such as the elderly,[63]may benefit from the promotion of memory Th17 cells.[64] Indeed, IL-17-producing $CD4^+$ T cells may also be increased in aged individuals indicating that these cells may have evolved for protection in this group.[65] Our own work suggests that vaccine-induced memory $CD4^+$ T cells rapidly produce IL-17 within 24 hours after pulmonary infection,[38] likely much sooner than vaccine-induced B cell responses. This is consistent with other studies that indicate that early production of IL-17 is protective during acute *P. aeruginosa* pulmonary infection.[66]

An additional potential benefit of a Th17-based vaccine is that a vaccine that relies on Th17 responses would, in contrast to B cell-mediated immunity, be independent of pathogen serotype.[67] This is crucial given the multitude of serotypes and phenotypic variability observed in *P. aeruginosa* clinical isolates.[68] Indeed, the Federal Hyperimmune Immunoglobulin Trial demonstrated no benefit to passive immunization with *P. aeruginosa* LPS O-antigen–specific IgG in critically ill adults.[69]

Route of vaccination

Successful vaccines have been developed against a number of mucosal pathogens and some studies demonstrate that immune responses are detectable at mucosal sites, including respiratory tissue, following systemic delivery of vaccines.[70–73] This body of work suggests that systemic immunization is adequate for protection against some mucosal pathogens, notably the influenza virus and the human papilloma virus,[73] and it is worth noting that almost every vaccine currently licensed in the United States is administered systemically via the intramuscular route.[74] Conversely, the lack of success seen in the development of vaccines against other mucosal pathogens, including *P. aeruginosa*, may in part be attributed to the inability of systemic immunization to adequately activate multiple arms of the innate and adaptive immune system and to target those responses to the infected mucosa. The field of *P. aeruginosa* vaccine research has responded to this challenge by utilizing novel vaccination routes shown to elicit mucosal immune responses, such as intranasal and intradermal immunization (Table 1). Evidence suggests that the route of immunization could influence different T cell effector functions in tissues proximal to the site of immunization[73] as well as different systemic and mucosal antibody responses.[75] Thus, the generation of mucosal immunity after systemic vaccination, such as intramuscular or subcutaneous immunization, may not recapitulate the immune responses induced after intradermal or intranasal vaccination with the same antigen/adjuvant formulation.[73] Intranasal immunization has been shown to result in the upregulation of Th17 cellular immunity in the lungs, independent of adjuvant choice[76] while intradermal immunization has been shown to induce the production of both IFN-γ and IL-17 in the lungs in a murine model of *P. aeruginosa*.[38]

Concluding remarks

The paucity of vaccines against mucosal pathogens, and particularly bacterial pathogens such as *P. aeruginosa*, highlights the need for new formulation and delivery strategies for eliciting local and mucosal cellular immunity. Directing vaccine-induced immune responses to the lung and other vulnerable tissues has proven to be a difficult task and as such, respiratory infections remain the leading cause of mortality in children under five.[77] Numerous recent studies have shown that Th17 $CD4^+$ T cells are important for vaccine-mediated immunity to pulmonary pathogens, including *P. aeruginosa, Mycobacterium tuberculosis, Bordetella pertussis, Streptococcus pneumoniae*, and *Klebsiella pneumoniae*.[67,78] There is a paradigm shift within the field of *P. aeruginosa* vaccine development towards the induction of multi-pronged immunity, including both humoral and cellular immunity,

particularly Th17-type $CD4^+$ T cell responses. In order to achieve this immunological response, a vaccine must induce both antibody and T cell memory responses within the tissues most vulnerable to infection. Current and future studies of *P. aeruginosa* vaccines should examine vaccination route and the addition of Th17-enhancing adjuvants to potential candidates. To identify immune correlates of protection, studies should include in-depth evaluation of effector and memory cellular immune responses to vaccination both systemically and at the site of infection.

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No potential conflicts of interest were disclosed.

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